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ORIGINAL PAPER

Ectopic expression of bacterial amylopullulanase enhances bioethanol production from maize grain

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Abstract
Key message **Heterologous expression of amylopullulanase in maize seeds leads to partial starch degradation into fermentable sugars, which enhances direct bioethanol production from maize grain.**
Abstract Utilization of maize in bioethanol industry in the United States reached ±13.3 billion gallons in 2012, most of which was derived from maize grain. Starch hydrolysis for bioethanol industry requires the addition of thermostable alpha amylase and amyloglucosidase (AMG) enzymes to break down the α-1,4 and α-1,6 glucosidic bonds of starch that limits the cost effectiveness of the process on an industrial scale due to its high cost. Transgenic plants expressing a thermostable starch-degrading enzyme can overcome this problem by omitting the addition of exogenous enzymes during the starch hydrolysis process. In this study, we generated transgenic maize plants expressing an amylopullulanase (APU) enzyme from the bacterium *Thermoanaerobacter thermohydrosulfuricus*. A truncated version of the dual functional APU (*TrAPU*) that possesses both alpha amylase and pullulanase activities was produced in maize endosperm tissue using a seed-specific promoter of 27-kD gamma zein. A number of analyses were performed at 85 °C, a temperature typically used for starch processing. Firstly, enzymatic assay and thin layer chromatography analysis showed direct starch hydrolysis into glucose. In addition, scanning electron microscopy illustrated porous and broken granules, suggesting starch autohydrolysis. Finally, bioethanol assay demonstrated that a 40.2 ± 2.63 % (14.7 ± 0.90 g ethanol per 100 g seed) maize starch to ethanol conversion was achieved from the *TrAPU* seeds. Conversion efficiency was improved to reach 90.5 % (33.1 ± 0.66 g ethanol per 100 g seed) when commercial amyloglucosidase was added after direct hydrolysis of *TrAPU* maize seeds. Our results provide evidence that enzymes for starch hydrolysis can be produced in maize seeds to enhance bioethanol production.

Keywords Amylopullulanase · Bioethanol · Starch · Transgenic maize

Introduction

The world's dependency on fossil fuels and the increasing demand for petroleum have raised concerns about future energy security. To reduce our dependence on fossil fuels, new forms of renewable energy need to be produced. Ideally, this new energy source would not only provide a solution to overcome an energy shortage but also create a cheaper and cleaner form of energy. Feedstocks that are useful for biofuel production, such as sucrose, starchy materials and cellulosic biomass, have been investigated through extensive research and their potential as a fossil fuel substitute has been explored by some countries (Hahn-Hägerdal et al. 2006; Balat and Balat 2009). Lignocellulosic

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biomass is the most abundant polymer on earth and recent research has been focused on cellulosic ethanol. However, hydrolysis of lignocellulosic materials is a slow, energy consuming and difficult process (Sanchez and Cardona 2008) even with recent improvements aimed at increasing cellulosic digestibility (Harrison et al. 2011; Brunecky et al. 2011; Zhang et al. 2012; Park et al. 2011; Heinonen et al. 2012). Starchy materials such as maize grain are still the most exploited polymers for biofuel production. In fact, maize-based ethanol production in the United States reached ±13.3 billion gallons in 2012 and 26 % of bioethanol was produced from maize grain, counting for 2.97 % of worldwide grain supplies (Anonymous 2013). This number is projected to be increased in the future.

Wet milling and dry grinding are the two main processes used to produce bioethanol from maize grain. The latter process is the most adopted strategy for bioethanol production in the US. Ethanol, thin silage and DDGS (distillers dry grain with sugar) are the main products of the dry grinding process (Bialas et al. 2010). Milled maize grain is gelatinized first at high temperatures in the presence of chemicals such as calcium oxide and ammonium to activate enzymes and adjust the pH. Alpha amylase enzyme is then added in the liquefaction process. The pH is lowered to the 4.2–4.5 range prior to the addition of the second enzyme, usually glucoamylase. Finally, the process is completed with fermentation to produce ethanol in the presence of nitrogen and urea. In theory, 718 l of ethanol per ton of starch is the expected outcome of this process (Castro et al. 2011).

Starch consists of two types of molecules: a linear glucose polymer linked with α-1,4 glucosidic bonds (amylose), and a highly branched glucose polymer that has α-1,6 branching linkages connecting linear chains (amylopectin). It has versatile applications in many industries such as in the sugar syrup, paper, and textiles industries (Muralikrishna and Nirmala 2005). Starch has a compact crystalline structure and is difficult to solubilize. Starch processing is carried out at high temperature to increase its solubility prior to enzyme digestions (Matsumoto 1982). Therefore, a thermostable starch-degrading enzyme is essential in this process. Conventional starch hydrolysis requires thermostable alpha amylase, usually from bacteria *Bacillus licheniformis* and *B. stearothermophilus*, to liquefy starch (Crabb and Mitchinson 1997). This process is performed at 105 °C to cut α-1,4 glucosidic linkages with its endo-acting mode of action (Singh and Soni 2001). Starch debranching enzyme, usually β-amylase, pullulanase or glucoamylase, is subsequently added at 60–65 °C for starch saccharification to further digest α-1,4 and α-1,6 linkages (Crabb and Mitchinson 1997; Matsumoto 1982). Starch is hydrolyzed into glucose before it can be converted into bioethanol through the fermentation process.

Amylopullulanase (APU) is a type II pullulanase enzyme bearing both the alpha amylase and pullulanase activities responsible for digesting the alpha 1,4 and 1,6 glucosidic linkages of starch, respectively (Vieille and Zeikus 2001). Molecular analysis of APU from bacteria *Thermoanaerobacter*, *Bacillus*, *Clostridium* and *Geobacillus thermoleovorans* NP33 showed a single active site for both alpha amylase and pullulanase activities (Saha et al. 1990, 1991; Shen et al. 1990; Nisha and Satyanarayana 2012); however, several aerobic bacteria such as *Bacillus circulans* F-2 and *Bacillus* sp. KSM1378 have different active sites for each activity (Sata et al. 1989; Hatada et al. 1996). Biochemical characteristics of this enzyme have been extensively studied (Saha et al. 1990, 1991). APU was mostly isolated from thermophilic bacteria with optimum enzyme activity at temperatures between 90 and 120 °C. A recent report demonstrated APU enzyme isolated from actinomycetes *Streptomyces erumpens* MTCC 7317 (Kar et al. 2008). The thermostability of APU, along with its activity at a broad pH range (pH 5.0–6.5, optimum pH 6.0), make it suitable for industrial applications (Vieille and Zeikus 2001).

Attempts to express starch-degrading enzymes in transgenic plants have been documented in several plant species. For example, bacteria alpha amylases have been produced in tobacco (Pen et al. 1992), narbon beans (Czihal et al. 1999) and sweet potato (Santa-Maria et al. 2011). Constitutive expression of thermophilic alpha amylase from *Thermotoga maritime* under 35S promoter activity caused conversion of starch into maltose (G2) after root homogenate was incubated at 80 °C for 14 h (Santa-Maria et al. 2011). Xu et al. (2008a, b) reported two transgenic rice lines expressing alpha amylase from *Bacillus stearothermophilus* and fungal glucoamylase from *Aspergillus awamori* in seeds. Their studies presented evidence that combination of the two transgenic rice lines could digest maize starch into glucose. On the other hand, a truncated form of the dual-function APU enzyme from *T. hydrosulfuricum* has been expressed in developing and germinated rice seeds under glutelin and alpha amylase promoters, respectively. Their work showed direct starch hydrolysis as well as enriched protein flour in rice, which is valuable for food industry (Chiang et al. 2005). These findings demonstrate that genetically engineered plants expressing starch hydrolyzing enzymes have potential to improve starch processing and can be useful for industrial applications.

Transgenic plants can be a cheap and practical tool for accumulating recombinant enzymes. Since maize grain is still the major crop utilized for bioethanol production, over expression of a starch-degrading enzyme in maize seeds can increase starch digestibility, avoid chemical pretreatments such as acid (Chung and Lai 2006) or protease (Pérez-

Carrillo et al. 2008), and make unnecessary the addition of commercial starch-degrading enzymes. The technology of transgenic plants expressing alpha amylase and APU has been patented (Lanahan et al. 2006, US Patent No.: 7,102,057 B2; Yu and Shaw 2004, US Patent No.: 6,737,563 B2), respectively. The patents describe that this technology is feasible to produce functional enzymes and allow self-starch processing in plants. Syngenta AG has also released and commercialized Enogen^{TM}, a maize hybrid expressing alpha amylase for efficient starch hydrolysis and higher bioethanol yields (http://www.syngenta.com/country/us/en/enogen/Pages/Home.aspx). Here, we report generating transgenic maize seeds overexpressing APU gene from *T. thermohydrosulfuricus* and utilizing these APU-expressing seeds for bioethanol production without adding external enzymes. Our study showed that incubation of the APU maize slurry at 80 °C resulted in starch digestion into smaller sugar molecules. Extended incubation for several hours followed by yeast fermentation resulted in the production of bioethanol. This finding is important for easier bioethanol production from maize grains and reducing the burden of the starch hydrolysis process.

Materials and methods

DNA construct

The APU gene of *Thermoanaerobacter thermohydrosulfuricus* is a 5 kb long gene. Site-directed mutagenesis study revealed that a 3-kb region (nucleotide 2899–5678) of the gene was essential for enzyme activity (Mathupala et al. 1993). The truncated APU (nucleotide 2899–5678 GenBank No. AAA23201) was codon optimized based on the codon usage frequencies for maize expression (http://www.kazusa.or.jp/codon/) and synthesized (Genscript, Piscataway, NJ). The vector construct of APU expression in maize endosperm is depicted in Fig. 1. APU expression was driven by the 27-kD γ zein endosperm-specific promoter (Marks et al. 1985). A FLAG epitope tag (Knappik and Pluckthun 1994) was fused in the N-terminal of APU and maize γ-zein signal peptide (Marks et al. 1985) was added in the 5′ end of the APU gene. C-terminal SEKDEL (ER-retention signal) (Munro and Pelham 1987) and VSP (soybean vegetative storage protein) terminator (Mason et al. 1993) were added downstream of the APU gene, respectively. A complete APU DNA cassette was cloned into the *Pst* I–*Eco*RI sites of pTF101.1 (Paz et al. 2004) binary vector containing *bar* gene conferring bialaphos (herbicide) resistance as a screenable marker for putative transgenic events to obtain the pNAP03 construct. The vector construct pNAP03 was transformed into *Agrobacterium tumefaciens* EHA101.

Maize transformation

Immature zygotic embryo of the Hi-II maize genotype was utilized in the transformation activity (Frame et al. 2002). Maize Hi-II immature embryos were infected with *A. tumefaciens* EHA101 carrying pNAP03 plasmid by the Plant Transformation Facility of Iowa State University. Bialaphos was applied to screen putative transgenic events. Transgenic plants were generated and advanced in the greenhouse to obtain T_1 seeds. APU transgenic maize was self-crossed or backcrossed with B73 for seeds propagation.

Southern blot analysis

Copy numbers of the APU transgene were determined by Southern blot. Genomic DNA was prepared from young leaves of T_1 plants as described by Murray and Thompson (1980). Aliquots (10 μg) of genomic DNA were digested with *Hin*dIII enzyme then digestion products were separated in 0.8 % agarose gel. Digested DNA was cross-linked into a membrane (Ambion® BrightStar®-Plus Positively Charged Nylon Membrane) using a cross-linker apparatus then pre-hybridized in Church's buffer for 2 h at 65 °C before the probe was added. An APU-specific probe was amplified from pNAP03 plasmid by PCR using forward (5′ TGTAGGTGATTTGCGTG 3′) and reverse (5′ TGTCTC TCCACCTTGACTCT 3′) primer pair (Fig. 1). The probe was labeled with ^{32}P dCTP and hybridized to the membrane, followed by an overnight incubation at 65 °C. The membrane was washed four times at 20-min interval with high stringency washing solution (0.2 × SSC, 0.1 % SDS) at 65 °C to remove non-specifically bound probe. The film was developed after 16 h exposure at −80 °C.

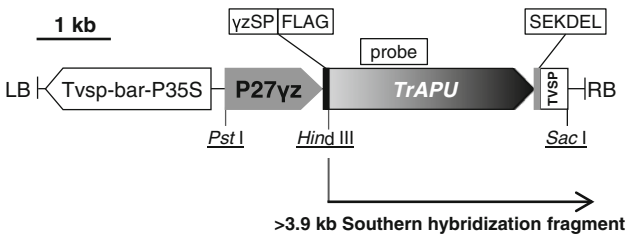

Fig. 1 Schematic diagram of T-DNA region in vector pNAP03. Codon-optimized *TrAPU* gene driven by 27-kD γ zein promoter (P27γz) was transformed into maize immature embryo via *Agrobacterium*-mediated transformation. *γzSP* maize γ-zein signal peptide, *FLAG* epitope tag, *SEKDEL* endoplasmic reticulum retention signal, *TVSP* soybean vegetative storage protein gene terminator, *bar* bialaphos resistance gene, *P35S* the cauliflower mosaic virus 35S promoter, *LB Agrobacterium* T-DNA Left border, *RB Agrobacterium* T-DNA right border, *Pst* I, *Hin*dIII, and *Sac* I, restriction enzymes, *kb* kilobases

RNA isolation and RT-PCR

APU transcription level was analyzed using reverse-transcriptase PCR (RT-PCR) method. Total RNA was extracted using Qiagen RNeasy Plant Mini kit (Qiagen Inc, Valencia, CA) following supplier's instructions. Complementary DNA (cDNA) was synthesized from 1 µg of the total RNA using SuperScript™ II reverse transcriptase (Invitrogen, Grand Island, NY) in a total volume of 20 µl. An aliquot of 2 µl cDNA (100 ng) was used as template for PCR to amplify approximately 800 bp of APU fragment using the same primer pair utilized for Southern blot analysis. Forward (5′ ATTCAGGTGATGGTGTGAGCCACAC 3′) and reverse (5′ GCCACCGATCCAGACACTGTACTTCC 3′) actin primers were used as internal control of housekeeping gene. Equal amounts of PCR product were loaded into each well and transcription level was determined semi-quantitatively.

Protein extraction and protein concentration determination

A single maize kernel was ground into powder using Genogrinder 2000 at 550 strokes/min for 90 s at the Iowa State University Plant Tissue and Seed Grinding Facility. Maize powder was resuspended in 10 µl of sodium phosphate buffer (pH 6.6) supplemented with protease inhibitor cocktail per mg of maize powder as described by Moeller et al. (2009). Sample was incubated with shaking at 37 °C for 2 h then centrifuged in a tabletop microcentrifuge at 13,000 rpm for 20 min. The supernatant was aliquoted into a new tube. Total soluble protein (TSP) concentration was measured with the Bradford method (Bradford 1976) using BSA (bovine serum albumin) as standard. Protein extract was used for the Western blot, enzyme activity and immunoprecipitaion assays.

Western blot

Aliquots (20 µg) of protein extract from a maize kernel were boiled for 5 min (Laemmli 1970). Samples were loaded into 4–15 % SDS gel (Bio-Rad) and separated at 110 V for 90 min. Western blot was carried out at room temperature unless otherwise stated. The gel was transferred onto a 0.2-µm nitrocellulose membrane using a semi-dry blot transfer apparatus (Bio-Rad, Hercules, CA) following manufacturer's instructions. The membrane was blocked with 5 % non-fat dry milk in phosphate buffered saline (PBS: 137 mM NaCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4) for 2 h followed by washing four times with PBST [PBS-0.05 % Tween-20 (v/v)]. The membrane was probed with a 1:5,000 dilution of anti-DYKDDDD monoclonal antibody produced in mice (Genscript, Piscataway, NJ). The membrane was incubated with horseradish peroxide (HRP)-conjugated goat anti-mouse IgG secondary antibody (Sigma Aldrich, St. Louis, MO) in a 1:5,000 dilution then washed four times with PBST. The membrane was incubated with sure blue TMB substrate (KPL, Gaithersburg, MD) for 20 min at room temperature for protein visualization.

Determination of APU activity

APU enzyme activity was measured with the dinitrosalycyclic acid (DNS) method (Miller 1959) as described by Mathupala et al. (1993). A mixture containing 40 µl of maize seed protein extract and 160 µl of 1.25 % pullulan (P4516, Sigma Aldrich, St. Louis, MO) in 50 mM acetate buffer (pH 6.0) containing 5 mM calcium chloride was incubated at 85 °C for 30 min. The reaction was stopped by adding 800 µl DNS solution to the mixture, and then the sample was boiled for 15 min and directly cooled down on ice. The samples were brought back to room temperature before measuring the absorbance at 635 nm. Enzyme activity was determined using glucose as standard. One unit of enzyme is defined as 1 µmole of glucose released per minute under assay conditions.

Purification of APU enzyme and enterokinase cleavage

Total protein extracted from 1 g of maize powder was freeze dried and diluted in 5 ml of Tris buffer saline (TBS: 50 mM Tris–Cl, 150 mM NaCl, pH 7.5). The FLAG-APU fusion protein was captured by passing the protein extract onto a chromatography column containing anti-DYKDDDDK IP resin (Genscript, Piscataway, NJ). The sample was incubated at 9 °C while gently shaking in a rotary shaker for 16 h. The next day, flow through was collected and the resin was washed four times with TBS. FLAG-APU bound to the resin was eluted in 5 M sodium chloride and concentrated using a microcon tube (EMD Milipore, Billerica, MA) then diluted in PBS. Purified FLAG-APU protein was tested for Western blot, enterokinase cleavage and enzyme activity analyses. Enterokinase cleavage was carried out by digesting 4.2 µg of FLAG-APU protein with 10 U of enterokinase (E4906, Sigma Aldrich, St. Louis, MO) in a total volume of 50 µl at room temperature for 16 h. Enterokinase was removed using trypsin inhibitor resin (T0637, Sigma Aldrich, St. Louis, MO) according to manufacturer's instructions. Purified APU both before and after enterokinase removal was utilized in enzyme assay and western blot.

Starch hydrolysis and scanning electron microscope (SEM)

Non-transgenic (negative segregant) and transgenic maize seeds were ground into powder as described above in the

protein extraction protocol. Acetate buffer containing 5 mM $CaCl_2$ (pH 6.0) was added to make a 1 % maize slurry. Samples were incubated at room temperature or 85 °C for 30 min. Starch was isolated using the protocol described by Li et al. (2008). Briefly, the samples were filtered through four layers of miracloth and the starch fraction was recovered by centrifugation (Avanti® J-E, Beckman Coulter) at 5,000*g* for 10 min. The starch pellet was resuspended in 0.1 M NaCl containing 10 % toluene and then vortexed for 1 h. This step was repeated two more times or until clean starch was obtained. Subsequently, the starch was washed three times with distilled water and two times with absolute ethanol. Starch samples were oven-dried at 40 °C for 48 h. Starch granule morphology was analyzed using an electron microscope at the Microscopy and Nanoimaging Facility of Iowa State University (ISU). Starch granules were mounted on silver tape and coated with gold:palladium (60:40) (Jane et al. 1994) then observed under digital JEOL 5800LV SEM at 1,500 and 5,000× magnitudes.

Starch hydrolysis and direct bioethanol production from TrAPU maize

Non-transgenic maize and *TrAPU* maize were ground into fine powder as described above. Starch content was measured beforehand using Total Starch (AA/AMG) kit from Megazyme (Bray, Ireland) following manufacturer's instructions. A total of 100 mg maize powder was suspended in 10 ml of sodium acetate buffer (pH 6.0) to a final concentration of 1 %. Starch hydrolysis products were analyzed using thin layer chromatography (TLC) assay. *TrAPU* maize slurry was incubated at 80 °C for different periods of time (1, 3, and 4 h or 4 h plus extended incubation at 30 °C for 72 h) and at 30 °C for 4 h. Non-transgenic maize was digested with commercial α-amylase (A3403, Sigma, St. Louis, MO) at 80 °C for 90 min followed by digestion with AMG (amyloglucosidase) (A7095, Sigma, St. Louis, MO) at 60 °C for 2 h or incubated at 80 °C for 4 h without enzymes addition. Measurement of bioethanol yield was carried out as follows: 1 g samples were incubated at 80 °C for 4 h with or without subsequent hydrolysis with 3 U of AMG for 2 h at 60 °C according to manufacturer's instructions. The process was continued by adding 0.03 % (w/w) ammonium sulfate and 0.02 g dry yeast *Saccharomyces cerevisiae* (Lesaffre Yeast Co., Milwaukee, WI). Fermentation was carried out at 30 °C for 72 h and bioethanol yield was measured using high-performance liquid chromatography (HPLC).

Statistical analysis

Statistical analysis was performed using JMP 10 software (SAS Institute Inc., Cary, NC, USA). Data were analyzed using analysis of variance. Comparison of APU activity between lines 27, 57 and 61 was analyzed using Student's *t* test. Comparison of reducing sugars concentration for bioethanol assay was analyzed with Duncan's multiple range test.

Results and discussion

Seed-specific expression of truncated (Tr)APU

Characterization of APU gene from *T. thermohydrosulfuricum* has been studied by Mathupala et al. (1993). Full-length APU encodes for a 5-kb long gene and consists of an N-terminal region for enzyme secretion and C-terminal region as a carbohydrate-binding domain. Truncation of this APU gene to 2,933 bp (from nucleotide 2899–5678) carrying a single active site for alpha amylase and pullulanase resulted in fully functional enzyme activity (Mathupala et al. 1993). A report by Lin et al. (2008) provided evidence that truncation of the C-terminal region of APU gene did not cause harmful effect to enzyme function.

In our study, the truncated APU (later referred as *TrAPU*) was codon optimized for maize expression. Codon bias should be carefully taken into account when expressing a prokaryotic gene in a eukaryotic system as it might affect protein translation efficiency (Campbell and Gowri 1990; Kurland 1991; Gustafsson et al. 2004). Codon-bias modification also has been shown to correlate with an increase in gene expression in plants. Higher accumulations of phytase were observed in *Brassica napus* transformed with codon-optimized *Aspergillus niger* phytase gene as compared to plants transformed with the native form the gene (Peng et al. 2006).

The APU expression cassette in maize (pNAP03) is depicted in Fig. 1. Heterologous *TrAPU* gene expression was regulated by maize 27-kD γ zein promoter and a soybean VSP terminator. Maize zein signal peptide was added to the 5′ end of the APU gene to target protein expression into the secretory pathway that has been shown to play role in recombinant protein accumulation in maize seeds (Yang et al. 2002; Moeller et al. 2009). We attached SEKDEL to the C-terminal of the *TrAPU* gene to retain the protein inside the endoplasmic reticulum (ER). This motif was sufficient to retain recombinant protein inside the ER and avoid further processing that might result in protein degradation (Munro and Pelham 1987). Previous studies showed enhanced recombinant protein expression when the gene of interest was fused with a KDEL motif (Spiegel et al. 1999; Peng et al. 2006). Marker peptide DY-KDDDDK (FLAG™) was added to facilitate recombinant protein detection. This peptide can be fused to the N- or

C- terminal of a protein. Its hydrophilic property exposes it to the surface, thus it does not interfere with the protein folding. This peptide can be cleaved by an enterokinase that recognizes the (DDDDK) amino acid sequence resulting in marker-free protein (Einhauer and Jungbauer 2001).

Vector construct pNAP03 was introduced into maize via *Agrobacterium*-mediated transformation. A total of 21 putative transgenic lines were generated. Enzymatic assay using DNS method, a method used for estimating reducing sugar, was carried out to screen T_1 generation. Out of 21 lines, 5 lines (14, 27, 47, 57, and 61) that performed high enzyme activity (Table 1) were advanced to generate T_2 seeds by self-pollination. Based on T_2 generation DNS assay data, three lines (27, 57, and 61) with the highest APU activities were chosen for further seeds production and analysis (data not shown). No noticeable phenotypical differences such as height or yield variance were detected in any of these lines (data not shown).

Agrobacterium-mediated transformation is a commonly used technique to transform plants because of its stability and low copy number transgene integration into the plant genome (Shou et al. 2004). A single copy transgene is desirable because multiple copy numbers often leads to silencing or partial transgene expression (Flavell 1994; Stam et al. 1997). Southern blot was performed to verify transgene integration and *TrAPU* transgene copy numbers. Based on seed quantities and viability, eight transgenic lines were selected for Southern blot analysis. Genomic DNA from young leaves of T_1 plants was digested with restriction enzyme *Hin*dIII. PCR-amplified ^{32}P-labeled *TrAPU* probe was used to hybridize the genomic DNA. Because *Hin*dIII cleaves only once in the T-DNA fragment (Fig. 1), transgene copy number can be estimated by the number of hybridization bands present on the autoradiography images. If the *TrAPU* gene is intact, the hybridization signals should have sizes larger than 3.9 kb. As can be seen from Fig. 2, the majority of events (6 out of 8) have low copy number (<3) transgene integration. Among them one event (line 78) has one single hybridization band greater than 8 kb. Line 12 showed one hybridization band smaller than 3 kb. It might be caused by partial transgene integration.

In this study, transgene copy number did not correlate with *TrAPU* expression (Table 1). Lines with multiple transgene copy numbers did not necessarily have high APU activity. For example, among eight Southern positive lines, the line with the highest APU activity is line 57 (8.82 U/μg TSP) with average specific activity of 3.05 ± 2.44 U/μg TSP. This line carries four transgene copies. Line 47 with 6 copies showed lower APU activity (2.37 ± 0.53 U/μg TSP) compared to line 57. Two 3-copy insertion lines (lines 12 and 60) have similar APU activities, 0.81 ± 0.23 and 1.24 ± 0.43 U/μg TSP, respectively. Three 2-copy

Table 1 Summary of transgene copy number and relative APU activity of T1 transgenic *TrAPU* maize lines

No	Event ID	APU specific activity (unit[a]/μg TSP) ± SD[b]	Southern copy #
1	4	1.94 ± 0.51	ND
2	5	0.99 ± 0.35	ND
3	6	2.03 ± 0.72	ND
4	7	2.82 ± 0.59	ND
5	11	3.62 ± 1.65	ND
6	**12**	**0.81 ± 0.23**	**3**
7	14	3.28 ± 1.80	ND
8	19	1.02 ± 0.37	ND
9	**27**	**2.34 ± 0.93**	**2**
10	**47**	**2.37 ± 0.53**	**6**
11	**51**	**0.75 ± 0.23**	**2**
12	53	1.19 ± 0.31	ND
13	**57**	**3.05 ± 2.44**	**4**
14	58	2.07 ± 0.32	ND
15	**60**	**1.24 ± 0.43**	**3**
16	**61**	**3.4 ± 2.06**	**2**
17	65	1.28 ± 0.22	ND
18	66	1.83 ± 0.32	ND
19	68	1.25 ± 0.28	ND
20	69	2.10 ± 0.52	ND
21	**78**	**1.95 ± 0.60**	**1**
22	WT	1.23 ± 0.37	0

WT non-transgenic maize B73 inbred, *ND* not-determined

[a] One unit of enzyme is defined as 1 micromole glucose liberated per minute under assay conditions, [b] SD is standard deviation of the mean of four determinations

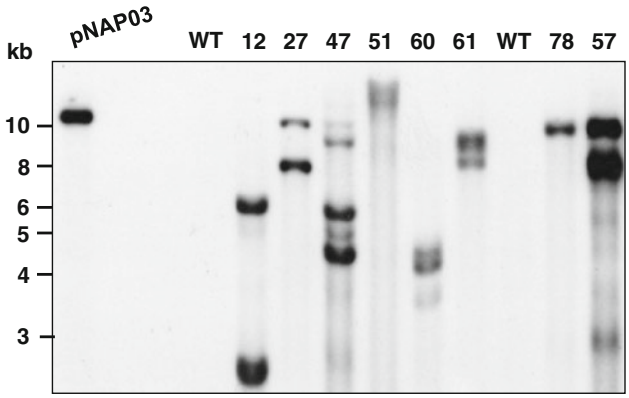

Fig. 2 Southern blot analysis of *TrAPU* transgenic maize (lines 12, 27, 47, 51, 60, 61, 78, and 57). Genomic DNA (10 μg) from eight bialaphos resistant lines and 75 pg of pNAP03 were digested with *Hin*dIII and separated on 0.8 % agarose gel. Digestion products were transferred into nitrocellulose membrane before being hybridized with *TrAPU* probe. pNAP03, *TrAPU* construct plasmid as positive control, *WT* non-transgenic B73 inbred line as negative control, *kb* kilobases

insertion lines (lines 27, 51, and 61) gave a wide range of activities between 0.75 ± 0.23 and 3.4 ± 2.06 U/μg TSP. The only single-copy event (line 78) had moderate activity, 1.95 ± 0.60 U/μg TSP. Similar observations have also been reported in transgenic maize expressing the fungal laccase enzyme (Hood et al. 2003). Transgenic plant with multiple transgenes did not necessarily have higher laccase compared to transgenic plant carrying a single copy transgene. On the other hand, T-DNA could be tandemly inserted into the same chromosome, which may lead to a silencing effect due to RNA duplex formation during transcription that triggers RNAi mechanism (Schubert 2006).

TrAPU expression in transgenic maize

Transcript level of *TrAPU* at different seed developmental stages was analyzed using RT-PCR. Immature maize seeds harvested at 10 and 20 DAP (day after pollination) and mature seeds from two potential lines (27 and 61) were analyzed. Total RNA isolated from individual transgenic kernel of lines 27 and 61 showed *TrAPU* transcription was highly present in immature seeds at 10 and 20 DAP and very low in mature seeds (Fig. 3a). This observation exhibited typical zein protein expression patterns. Zein is a major storage protein in maize and makes up 50–60 % of maize total protein (Monjardino et al. 2006). Accumulation of this protein started at 10–12 DAP and achieved its maximum level at 18–22 DAP (Marks et al. 1985). Typically the zein transcripts are high in immature seeds but reduced gradually during the maturation process (Marks et al. 1985; Woo et al. 2001). The reduced *TrAPU* transcript levels observed in the mature seeds here are likely due to the translation of transcripts into protein.

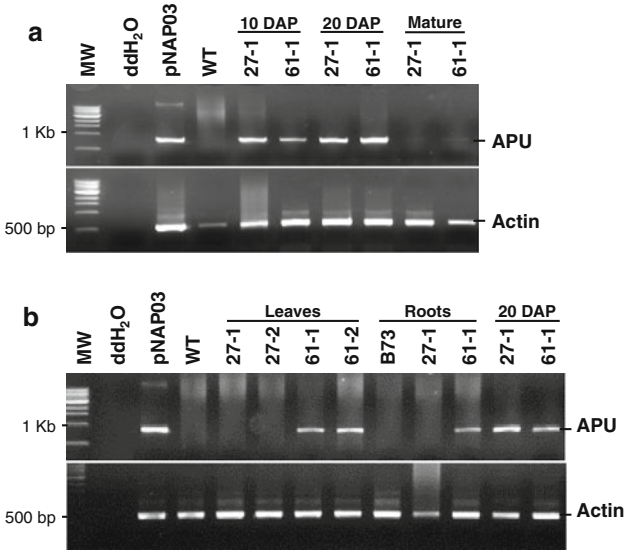

Fig. 3 Reverse-transcriptase PCR analysis of two *TrAPU* transgenic maize (lines 27 and 61) at different developmental stages (**a**) and different organs (**b**). RNA was extracted from 1 to 2 samples of immature seeds (10 and 20 DAP), mature seeds (Mature), leaves or roots. Actin primer is utilized as internal control. *MW* molecular weight marker, *ddH$_2$O* distilled water as template of negative control, *pNAP03*, *TrAPU* construct plasmid control, *WT* mature seed of non-transgenic maize inbred B73, *DAP* days after pollination

Analysis of *TrAPU* expression was also conducted in different tissues by comparing the transcript level in leaves, roots, and seeds that were sampled from the same plant. For line 27, *TrAPU* transcripts can only be detected in immature seeds, not in leaves or roots, as expected (Fig. 3b). However, in line 61 *TrAPU* expression can be observed in all tissues tested, which is contrary to the native activity of an endosperm-specific promoter. This discrepancy has also been observed in transgenic plants driven by the 27-γ zein promoter as studied by Russell and Fromm (1997). In that study, 5 out of 17 maize transgenic lines tested showed non-endosperm transgene expression (including leaf, pollen, embryo and maternal seed tissue). One possible explanation is that the transgene might be inserted behind regulatory elements that override the tissue-specific promoter (Mark Tucker, personal communication).

APU protein production was detected using western blot against an anti-DYKDDDDK antibody (Fig. 4). Two PCR-positive kernels from each line (27, 57, and 61) were examined. Equal amount of total protein extracted from single kernel (20 μg) was loaded into SDS-PAGE gel. Figure 4 shows that APU protein expression varied among lines. Line 57 showed the highest APU, while line 27 had the lowest APU level.

To further examine the transgenic lines, enzymatic activity of APU was determined using DNS assay under optimum conditions at 85 °C for 30 min (Mathupala et al. 1993). Three transgenic lines, 27, 57 and, 61, were tested and non-transgenic B73 maize was utilized as a negative control. Only PCR-positive seeds were pooled and used for the analysis. The APU-specific activity of each line was the average specific activity of at least eight positive single kernels from three technical replications. Similarly, the enzyme-specific activity was different between transgenic lines (27, 57, and 61) indicating variability of APU accumulation in maize seeds (Fig. 5). Line 57 showed the highest specific activity (23 U/μg TSP) and line 27 showed

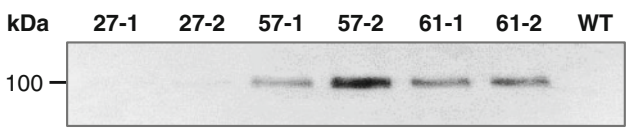

Fig. 4 Immunoblotting analysis of *TrAPU* transgenic maize (lines 27, 57, and 61). Two single kernels were analyzed from each line. Total protein (20 μg) extracted from single mature kernel was loaded into each well. APU protein was detected using anti-DYKDDDDK tag antibody. *kDa* kilodalton, *WT* B73 non-transgenic maize inbred

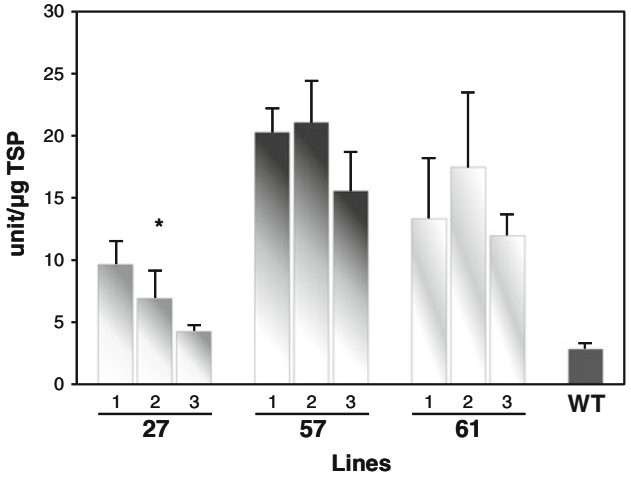

Fig. 5 APU enzyme specific activity of *TrAPU* transgenic maize (lines 27, 57, and 61). Three ears from each line were analyzed. Each ear represents a pool of at least 8 T3 generation transgenic seeds. *WT* non-transgenic maize B73 inbred. Protein extract mixed with 1.25 % pullulan substrate was incubated at 85 °C for 30 min. One unit of enzyme is defined as 1 micromole of glucose liberated per minute under assay conditions. *Asterisk* (*) indicate statistically different at ($P < 0.05$). *Error bars* represent standard deviation of mean from three technical determinations

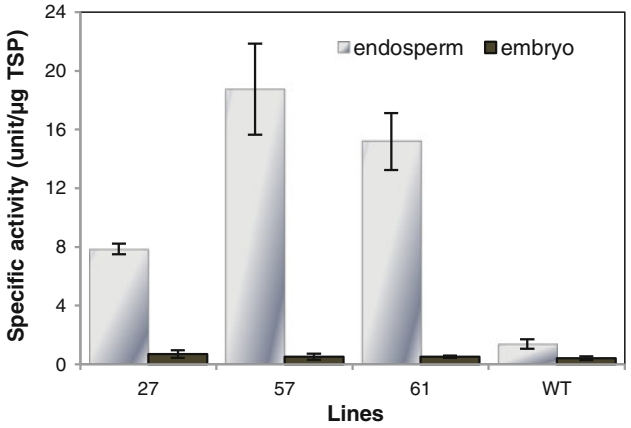

Fig. 6 Accumulations of TrAPU enzyme in endosperm and embryo of mature seed from three transgenic maize lines (27, 57, and 61). *WT* pool of non-transgenic segregants maize seeds from lines (27, 57, and 61); *TSP* total soluble protein. *Error bars* represent standard deviation of mean from 3 technical determinations

the lowest APU activity. Comparison of the APU activity between line 27 and lines 57, and 61 is statistically significant ($P < 0.05$). No significant difference was observed between lines 57 and 61. There was negligible enzyme activity in non-transgenic maize (B73). This result showed that protein expression is correlated with enzyme activity. The high enzyme activity observed in line 57 was correlated with the high accumulation of the APU protein in this line. On the other hand, the low enzyme activity of line 27 was correlated with the low APU protein accumulation.

Chiang et al. (2005) showed that the expression of truncated APU in transgenic rice seeds led to the autohydrolysis of starch into glucose at high temperature. They reported that the highest APU-specific activity in rice seeds was 160 U/min/µg TSP. This is approximately seven times higher than what we observed in maize seeds. One possible explanation could be the different starch properties and gelatinization temperatures (GT) of the maize and rice grains used in these studies. In general, maize has onset GT at 58.3 °C and peak temperature at 83 °C (Tester and Morrison 1990). On the other hand, gelatinization in rice seeds [Tainung 67, (Chiang et al. 2005)] generally occurs at 55–80 °C (Huang et al. 2009). The APU assays in Chiang's work and this study were performed at 85 °C. It is speculated that more starch molecules were gelatinized and available for enzyme attack in rice grains than in maize grain at this temperature, which led to a higher APU-specific activity value in rice.

Tissue specificity of the 27-kD γ zein promoter in seeds has been studied previously. Heterologous expression of *E. coli* heat-labile enterotoxin (LT-B) driven by this promoter demonstrated recombinant protein accumulation in the starch granules of maize kernels (Chikwamba et al. 2003). To examine the main accumulation of the APU enzyme in transgenic maize lines used in this study, 20 maize kernels of lines 27, 57, and 61 were dissected and separated between germ and endosperm. Negative segregant from each line was utilized as negative control. Protein was extracted from both fractions and enzyme activity was measured. Results showed that APU activity was mainly observed in the endosperm fraction indicating its accumulation in this tissue (Fig. 6). It is interesting to note that no APU activity was detected in embryo fraction of line 61, even though APU transcripts were found in its leaf and root (Fig. 3b). Because the leaf and root expression of APU transcripts, one would expect some level of APU enzymatic activity in embryos if the 27-kD γ zein promoter lost its endosperm specificity. Further investigation is required to understand this phenomenon.

FLAG™ did not interfere with TrAPU enzyme activity

The fusion tag known as FLAG™ is a water soluble antigenic marker peptide consists of eight amino acids (AspTyrLysAspAspAspAspLys/DYKDDDDK) that can be digested by protease to generate marker-free protein (Einhauer and Jungbauer 2001). The APU enzyme was purified using immunoprecipitation method. Total protein (crude extract) isolated from seeds of line 57 (Fig. 7, lane 3) was loaded onto a column containing anti-DYKDDDDK agarose beads. The flow through contained unbound APU protein (Fig. 7, lane 2). Pure APU was pulled down from the resin and concentrated. Each fraction was tested using western blot and enzyme activity assays (Fig. 7; Table 2).

A total of 6.4 % of the APU enzyme was recovered (Fig. 7, lane 6) with a specific activity of 105.5 Unit/μg TSP or ten times higher compared to the crude extract. Purified fusion FLAG-APU protein was digested with enterokinase (Fig. 7, lane 4) to test the effect of the FLAG tag on APU enzyme activity. As can be seen in Fig. 7 (lanes 4, 5 and 7), no APU band was detected using anti-FLAG antibody in Western analysis in samples treated with enterokinase, suggesting that FLAG was no longer attached to the APU enzyme. When FLAG-removed APU sample (Fig. 7, lane 7) was measured for APU enzyme activity, the assay showed that similar values of specific activity were obtained (Table 2). These results suggest that FLAG does not interfere with enzyme activity.

Scanning electron microscope

To examine whether spontaneous starch hydrolysis actually happened in APU maize, we performed SEM analysis on starch granules from both *TrAPU* and non-transgenic maize seeds after incubation at room temperature (22 °C) or 85 °C for 30 min. Starch granules are packed tightly in grains. Heat treatment or gelatinization can break the granules physically and open them for enzyme attack. Enzyme treatment on starch granules causes their degradation (Singh and Soni 2001). Damaged granules can be observed under such conditions (Naguleswaran et al. 2012).

As can be seen in Fig. 8a and d, starch granules of *TrAPU* (line 57) and non-transgenic maize at room temperature had an irregular and polygonal shape; similar observations were also reported by Naguleswaran et al. (2012). However, high temperature treatment at 85 °C of these starch samples showed that most granules of *TrAPU* maize were digested and some were broken, suggesting there was starch hydrolysis (Fig. 8e, f). On the other hand, heat treatment on starch granules of non-transgenic maize caused their shape deformation, but broken granules were hardly seen (Fig. 8b, c). The same observation has been previously reported (Chung and Lai 2006; Jane et al. 1994). In these studies, maize starch treated with glucoamylase at 50 °C caused erosion on granule surface. Longer incubation time showed a more damage on granule surface (Naguleswaran et al. 2012).

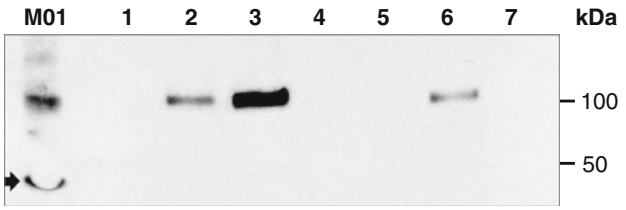

Fig. 7 Western analysis of TrAPU purified by FLAG-epitope based immunoprecipitation using anti-FLAG antibody. Lane M01, A 52-kDa multiple tagged fusion protein (arrow head) as positive control (M0101, Genscript, Piscataway, NJ, USA); *Lane 1* protein extract of non-transgenic B73 inbred, *Lane 2* recovered liquid sample (flow through) passing through the FLAG column, *Lane 3* crude protein extract of *TrAPU* seeds, *Lane 4* FLAG column purified protein sample digested with enterokinase, *Lane 5* FLAG column elution sample after enterokinase cleavage, *Lane 6* FLAG column purified protein, *Lane 7* recovered liquid sample passing through FLAG column after FLAG removal with enterokinase. *kDa* kilodalton

Table 2 APU purification and enterokinase cleavage

Samples	Yield (%)	Glucose (mM)	Unit[a]	Specific activity (unit/μg) TSP
Crude extract	100	21.84	728.03	11.03
Flow through	90	10.33	344.24	5.74
FLAG-APU	6.4	13.29	443.07	105.5
APU after FLAG removal	–	12.81	427	101.65
FLAG elution	–	–	–	–
WT	–	1.36	45.2	1.03
Positive control*	–	5.02	167.4	3.28

[a] One unit of enzyme is defined as 1 micromole glucose liberated per minute under assay conditions

* Pullulanase purchased from Sigma was used as positive control

Sugar product analysis of TrAPU maize

To evaluate whether transgenic TrAPU maize seeds can be used directly for bioethanol production, we performed direct hydrolysis and analyzed the sugar products with TLC. Because APU possesses dual enzymatic activities, we expected that its amylase part can hydrolyze the α-1,4 bonds and its pullulanase part can hydrolyze α-1,6 glycosidic bonds to facilitate the production of glucose (G1), maltose (G2), or maltotriose (G3) (Mathupala et al. 1990). In this experiment, we included non-transgenic maize seeds that were incubated with the commercial grade enzymes α-amylase and amyloglucosidase (AMG), two commonly used enzymes in the starch degradation process, as our positive control.

As shown in Fig. 9 (lane 3), the incubation of *TrAPU* maize line 57 at 80 °C for 1 h without addition of any external enzyme led to an autonomous starch digestion process. Most products were sugar molecules G1–G4 (glucose, maltose, maltotriose and maltotetraose). Maize-derived APU action was more efficient after 60 min incubation (lane 3) compared with non-transgenic maize hydrolyzed with commercial α-amylase for 90 min (lane 1). The non-transgenic maize hydrolyzed with commercial α-amylase produced a wide spectrum of oligosaccharides and dextrin larger than G4 (lane 1), which cannot be utilized by yeast for ethanol fermentation. There was also a substantial amount of starch not being hydrolyzed, which remained at the origin.

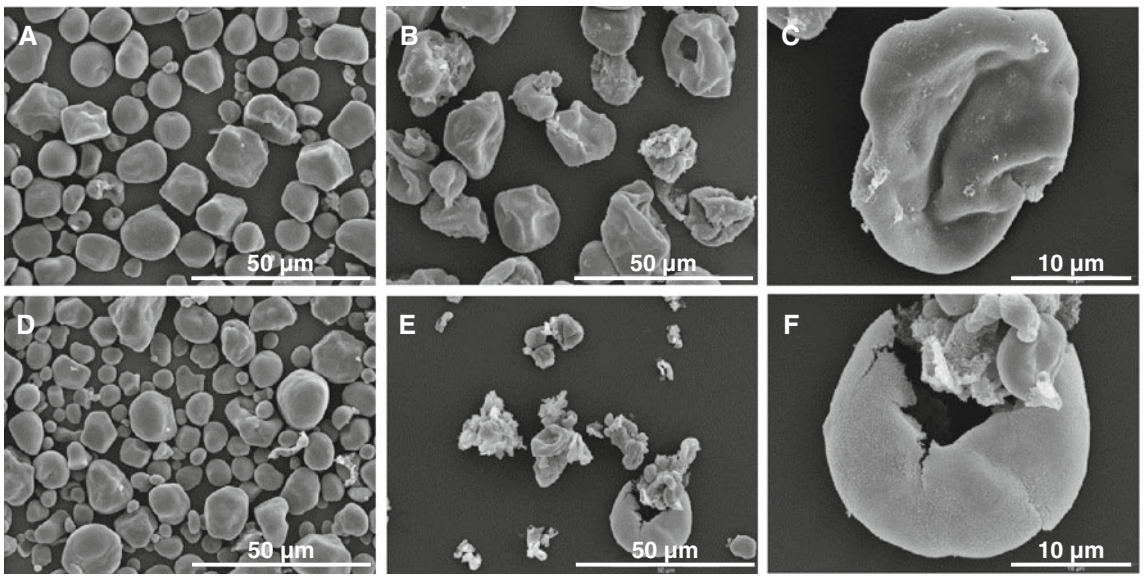

Fig. 8 Scanning electron micrograph of starch granules isolated from maize mature seeds. Non-transgenic maize starch samples were treated at room temperature (**a**), or at 85 °C (**b**, **c**) and *TrAPU* maize starch samples at room temperature (**d**) or at 85 °C (**e**, **f**). *Scale bar* 50 µm (×1,500) for panels **a**, **b**, **d** and **e**; 10 µm (×5,000) for panels **c** and **f**

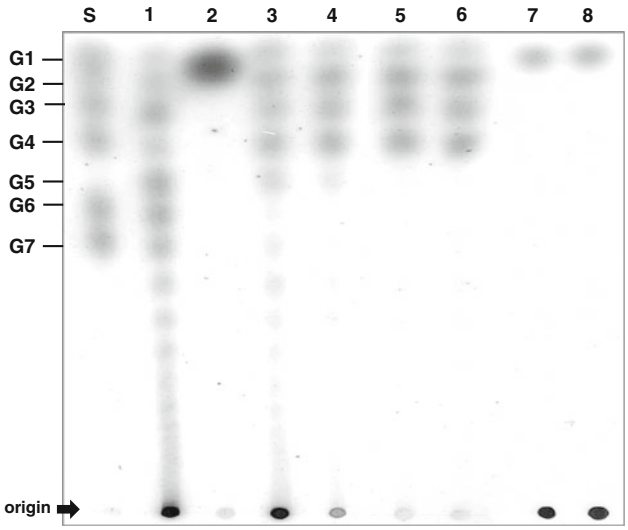

Fig. 9 TLC analysis of *TrAPU* maize hydrolysis products. 1 % of wild type (WT) maize and *TrAPU* maize slurry was used in the experiment. *Lane S* standard, *Lane 1* WT plus α-amylase, 80 °C, 4 h, *Lane 2* WT plus α-amylase (80 °C, 4 h) and AMG (60 °C, 2 h), *Lane 3* TrAPU, 80 °C, 1 h, *Lane 4* TrAPU, 80 °C, 3 h, *Lane 5* TrAPU, 80 °C, 4 h, *Lane 6* TrAPU, 80 °C, 4 h, then 30 °C, 72 h, *Lane 7* TrAPU, 30 °C, 4 h, *Lane 8* WT, 80 °C, 4 h. *G1* glucose, *G2* maltose, *G3* maltotriose, *G4* maltotetraose, *G5* maltopentaose, *G6* maltohexaose, *G7* maltoheptaose

On the other hand, the *TrAPU* maize was not as efficient as the non-transgenic maize that was hydrolyzed with both α-amylase and AMG (Fig. 9, lane 2). Complete conversion of starch into glucose (G1) was observed in this sample. AMG cleaves starch from the non-reducing end releasing glucose (Saha and Zeikus 1989). Starch hydrolysis of *TrAPU* maize was inefficient at 30 °C suggesting that APU enzyme had little activity at this temperature (Fig. 9, lane 7). Non-transgenic maize, as a negative control (Fig. 9, lane 8), showed that little starch was hydrolyzed after being incubated at 80 °C for 4 h. G1 spots observed in *TrAPU* maize incubated at 30 °C (Fig. 9, lane 7) and non-transgenic maize incubated at 80 °C (Fig. 9, lane 8) are likely products of endogenous starch hydrolysis activity in these samples.

Longer incubation times (up to 4 h) of *TrAPU* maize (Fig. 9, lanes 3–5) showed that more starch molecules were converted into G1–G4 as indicated by the disappearance of sugar molecules larger than G5 and diminishing of undigested starch spot at the origin. Extended incubation for 72 h at 30 °C after the 80 °C incubation for 4 h did not give noticeable difference (Fig. 9, lane 6). Reducing sugar content analysis (Table 3) showed that *TrAPU* maize produced higher reducing sugar concentration (29.6 ± 0.26 mg/100 mg maize) as compared with non-transgenic maize (8.1 ± 0.07 mg/100 mg maize) when they were both incubated at 80 °C. Addition of AMG in the *TrAPU* maize reaction resulted in reducing sugar concentration (64.3 ± 0.15 mg/100 mg maize) that was nearly the same with non-transgenic maize degraded with AMG (64.1 ± 0.13 mg/100 mg maize).

Studies on enzyme hydrolysis pattern of the APU from *Clostridium thermohydrosulfuricum* strain 39E (Saha et al. 1991) and *Thermoanaerobacter* strain B6A (Saha et al. 1990) showed glucose molecules and G2–G4 as the main products of starch hydrolysis from different substrates (amylose; amylopectin; and soluble starch). Characterization of APU

Table 3 Direct bioethanol production from *TrAPU* maize

Samples	Starch content (%)	Reducing sugars (mg/100 mg)	Ethanol yield (g/100 g)	Conversion efficiency[e] (%)
WT	66.1 ± 0.50	8.1 ± 0.07[a]	0.02 ± 0.01[a]	0.8 ± 0.29[a]
WT + 3u AMG	66.1 ± 0.50	64.1 ± 0.13[c]	32.7 ± 0.52[c]	87.2 ± 1.62[c]
TrAPU maize	64.5 ± 0.39	29.6 ± 0.26[b]	14.7 ± 0.90[b]	40.2 ± 2.63[b]
TrAPU maize + 3u AMG	64.5 ± 0.39	64.3 ± 0.15[c]	33.1 ± 0.66[c]	90.5 ± 1.69[d]

The values with different superscripts within a column are significantly different from each other ($P < 0.05$) by Duncan's multiple range test. Identical superscript indicates no significant difference

[e] Conversion efficiency (%) = 100 × ethanol yield/starch content/theoretical yield of ethanol (56.73)

WT wild type Hi-II maize

from *Geobacillus stearothermophilus* demonstrated maltose and maltotriose (G2–G3) were produced from starch (Zareian et al. 2010). In this study, sugar molecules G2–G4 were also major products produced during *TrAPU* maize starch hydrolysis. Liquefaction of transgenic rice seeds expressing alpha amylase from *B. stearothermophilus* resulted in G5 sugar as the major product while G1–G3 were also detected (Xu et al. 2008a). Most of the sugar molecules were nearly converted into G1 when transgenic rice seeds expressing glucoamylase from *A. awamori* were added in the reaction (Xu et al. 2008b).

Maize-derived APU enhances bioethanol production

Starch hydrolysis was followed by yeast fermentation to determine bioethanol production from *TrAPU* maize. Bioethanol yield measured by HPLC showed that *TrAPU* maize alone, without adding other enzymes, produced 14.7 ± 0.90 g ethanol/100 g of maize or a 40.2 ± 2.63 % ethanol conversion of maize starch (Table 3). Yeast was able to effectively use G1 and G2 for fermentation but did not as efficiently using G3, and could not utilize G4 (Nout and Bartelt 1998; Zastrow et al. 2001). It is likely that G2 and G3, major products of APU hydrolysis, were utilized by yeast to produce bioethanol from the corn sample. On the contrary, non-transgenic maize produced little ethanol (0.02 ± 0.01 g ethanol/100 g of maize) when treated under the same condition. The bioethanol yield of *TrAPU* maize was about half of the bioethanol produced using non-transgenic maize hydrolyzed with AMG (32.7 ± 0.52 g ethanol/100 g of maize, 87.2 ± 1.62 % conversion). Addition of AMG to the *TrAPU* maize increased ethanol yield and conversion efficiency to 33.1 ± 0.66 g ethanol/100 mg maize and 90.5 ± 1.69 %, respectively.

Conclusion

Starch is an important feedstock for bioethanol production. Starchy biomass is still preferred over cellulose because of its abundance and established processing. However, large amounts of amylase enzyme and energy are needed during starch hydrolysis, which form the main avenues for improvement. One way to solve this problem is to express a starch-degrading enzyme in starch accumulating crop so the external enzymes needed for the hydrolysis could be reduced or omitted.

Our study provided evidence that expression of a truncated version of the bacterial *T. thermohydrosulfuricus* APU enzyme in maize seed can enhance starch processing efficiency for bioethanol production. While our current expression level and experimental design does not provide 100 % ethanol conversion, this study demonstrated the feasibility of such an approach. Future research on enhanced APU production and simultaneous saccharification fermentation may further improve bioethanol yield.

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